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Co-administration of rofecoxib and tramadol results in additive or sub-additive interaction during arthritic nociception in rat

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Abstract

Over the decades, nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids are the most commonly used analgesics in the management of acute and chronic pain. In order to assess a possible antinociceptive interactions, the antinociceptive effects of rofecoxib p.o., a preferential inhibitor of cyclooxygenase-2, and tramadol-hydrochloride p.o., an atypical opioid analgesic, administered either separately or in combination, were determined using a rat model of arthritic pain. The data were interpreted using the surface of synergistic interaction (SSI) analysis and an isobolographic analysis to establish the nature of the interaction. The SSI was calculated from the total antinociceptive effect produced by the combination after subtraction of the antinociceptive effect produced by each individual drug. Female rats received orally rofecoxib alone (1.0, 1.8, 3.2, 5.6, 10.0, 17.8, 31.6 and 56.2 mg/kg), tramadol alone (1.8, 3.2, 5.6, 10.0, 17.8, 31.6 and 56.2 mg/kg) or 12 different combinations of rofecoxib plus tramadol. Five combinations exhibited various degrees of sub-additive (i.e. less than the sum of the effects produced by the each drug alone) antinociceptive effects (3.2 mg/kg tramadol with 7.8 mg/kg rofecoxib; 5.6 mg/kg tramadol with either 10.0 or 17.8 mg/kg rofecoxib; 10.0 mg/kg tramadol with either 10.0 or 17.8 mg/kg rofecoxib), whereas the other 7 combinations showed additive antinociceptive effects (i.e. the sum of the effects produced by each agent alone). Three combination of rofecoxib+tramadol (10.0+5.6, 10.0+10.0, and 17.8+5.6 mg/kg respectively) presented high sub-additive interactions ($P<0.002$: $Q=9.5$). The combination rofecoxib (17.8 mg/kg)+tramadol (10.0 mg/kg) caused gastric injuries less severe than those observed with indomethacin, but more severe than those obtained with rofecoxib or tramadol in single administration. The antinociceptive interaction of rofecoxib and tramadol suggests that combinations with these drugs may have no clinical utility in pain therapy.

Keywords: Inflammatory pain; Cyclooxygenase; Rofecoxib; Tramadol; Synergism

1. Introduction

In the treatment of clinical pain, the choice of a specific analgesic drug is, in general, made on the basis of the type of pain. The opioid analgesic drugs remain the most effective therapy available for the treatment of moderate to severe pain; however, the problems arising from unwanted side-effects persist. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is limited by ceiling effects and by adverse events, the most clinically important of which are upper gastrointestinal side-effects, such as dyspepsia, peptic ulceration haemorrhage, and perforation, leading to death in some patients (Griffin, 1998). The development of new pain management strategies affords clinicians with additional treatment options for pain management. Preoperative administration of some analgesics, for example, has been shown to reduce the onset of postoperative pain. Another approach involves combining

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analgesics that target both peripheral and central pain pathways to deliver comparable analgesia at lower – and hence more tolerable – doses of the component drugs. Combining analgesic drugs with different site of action, modes of action, onset and duration, can greatly enhance their capacity to minimize pain, be tolerated better and reduce recovery time (Mehlisch 2002). Therefore, the combinations of opioids and NSAIDs are commonly used to control postoperative pain (Wideman et al., 1999; Picard et al., 1997). The potential advantage of using combination therapy is that analgesic effects can be maximized while the incidence of adverse side-effects can be minimized (Picard et al., 1997). Therefore, using combinations of medications that offer analgesic synergism should allow a reduction in required dosage and decrease the incidence of adverse effects (Wei-wu et al., 1999).

On the other hand, while clinical studies with NSAIDs and opioids suggest an additive or possibly synergistic interaction, few quantitative studies to establish the antinociceptive interaction have been conducted. Quantifying an antinociceptive synergistic effect presents practical and ethical limitations in human subjects, but adequate animal models of nociception have been described. There are combinations of opioids and NSAIDs, which have positive synergistic interactions (Wideman et al., 1999; Picard et al., 1997: Wei-wu et al., 1999), but only few of these have been analysed in preclinical models (Maves et al., 1994; Sandrini et al., 1998, 1999; Taylor et al., 1998). Our group has addressed the analysis and evaluation of interactions between opioids and NSAIDs (López-Muñoz et al., 1993a, 1994a,b; López-Muñoz, 1994; Salazar et al., 1995; Déciga-Campos et al., 2003; López-Muñoz et al., 2004).

Rofecoxib is a NSAIDs, preferential COX-2 inhibitor, as observed in numerous in vitro and in vivo assays (Chan et al., 1999), developed to treat osteoarthritis, acute pain conditions, and dysmenorrhoea. Worldwide, over 80 million people were prescribed rofecoxib at some time. On September 30, 2004, Merck voluntarily withdrew rofecoxib from the market because of concerns about increased risk of heart attack and stroke associated with long-term, high-dosage use (Bombardier et al., 2000). Tramadol-hydrochloride is a centrally acting agent generally classified as a synthetic opioid analgesic, but the mechanism of action is not completely understood. These considerations determined the principal objective of the present study: to investigate the antinociceptive effect of rofecoxib (because is a classical preferential COX-2 inhibitor) and tramadol (because is an atypical opioid) by administration alone or in combination, using the pain-induced functional impairment model in the rat (PIFIR model), an animal model of arthritic pain (López-Muñoz et al., 1993b).

2. Materials and methods

2.1. Animals

Female Wistar rats [Crl (WI) BR] weighing 180–200 g, were used in this study. The experiments were performed in female animals because all of our previous experimental results (when were used combinations) had been reported using female rats; in addition, it is necessary to study the antinociceptive effects in female subjects too (López-Muñoz et al. 1993a,b; López-Muñoz, 1994, 1995; López-Muñoz et al. 1994a,b, 1995, 2004; Hoyo-Vadillo et al., 1995; López-Muñoz and Salazar, 1995; Salazar et al., 1995; López et al., 2006). Food was withheld 12 h before the experiments, with free access to water. All experimental procedures followed the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Covino et al., 1980) and the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983), and were carried out according to a protocol approved by the local Animal Ethics Committee. The number of experimental animals was kept to a minimum, and animals were housed in a climate- and light-controlled room with a 12-h light/dark cycle. All animals were acclimatized to laboratory environment for at least 12 h before testing.

2.2. Drugs

Uric acid (Sigma, St. Louis, MO, USA) was suspended in mineral oil; rofecoxib was obtained from Merck Sharp & Dohme (Mexico City, Mexico) and tramadol-hydrochloride was obtained from Laboratories RIMSA (Mexico City, Mexico). All the drug solutions for oral administration (rofecoxib, tramadol and indomethacin) were freshly prepared by suspending them in 0.5% carboxymethylcellulose, and administered 4 ml/1000 g body weight.

2.3. Measurement of antinociceptive activity

Antinociceptive activity was assessed using the PIFIR model, which has been described in detail (López-Muñoz et al., 1993b). The animals were anaesthetised with ether in an anaesthesia chamber (Pyrex glass dryer saturated with ether vapor). Nociception was induced by an intra-articular (i.a.) injection of 0.05 ml of 30% uric acid suspended in mineral oil in the knee joint of the right hind limb. The suspension was prepared by grinding 3.0 g of uric acid with 10 ml of mineral oil in a glass mortar and pestle (Pyrex). The intra-articular injection was performed through the patellar ligament using a 1 ml glass syringe (Beckton, Dickinson LTDA, Brazil) with a 24-gauge needle of 5 mm. Immediately afterwards an electrode was attached to the plantar surface of each hind paw (right and left) between the plantar pads . The rats were allowed to recover from anaesthesia and were then placed on a stainless steel cylinder of 30 cm diameter, which was rotated at 4 rpm, forcing the rats to walk for periods of 2 min every 30 min for 6.5 h. Training periods were not necessary because the rats learned in the first minutes. The time of contact between each electrode on the limbs of the rat and the cylinder was recorded with a computer, this being the variable measured. When the electrode placed on the animal's paw made contact with the cylinder floor, a circuit was closed and the time that the circuit remained closed was recorded. After uric acid injection, the rats developed progressive dysfunction of the injured limb. The time of contact of the injured hind limb reached a zero value 2.5 h after the injection of uric acid; at this time, rofecoxib

and tramadol were administered either alone or in combination. This time was considered as time zero for measurement of antinociceptive effects, and these effects were measured every 30 min for the next 4 h. This permitted determination of the time course of the antinociceptive effects in the same animal. Antinociception was estimated as recovery of the time of contact. The data are expressed as the functionality index percent (FI%, i.e., the time of contact of the injected foot divided by the time of contact of the control left foot multiplied by 100). For the purpose of this study, inducing nociception in the experimental animals was unavoidable. However, care was taken to avoid unnecessary suffering. All experiments were performed between 7:00 a.m. and 2:00 p.m.

2.4. Study design

The antinociceptive effects produced by rofecoxib p.o. and tramadol p.o. given either individually or in combination were studied. First, each dose of rofecoxib (1.0, 1.8, 3.2, 5.6, 10.0, 17.8, 31.6 or 56.2 mg/kg) or tramadol (1.8, 3.2, 5.6, 10.0, 17.8, 31.6 or 56.2 mg/kg) was given to six animals to obtain the corresponding dose–response curves and the doses of rofecoxib (3.2, 5.6, 10.0 or 17.8 mg/kg) and tramadol (3.2, 5.6 or 10.0 mg/kg) were then combined (p.o.) to analyze possible synergistic interactions (12 combinations in total). Subsequently, rofecoxib (10.0 and 17.8 mg/kg) with tramadol (31.6 mg/kg) were included as 2 new combinations. Adequate controls were performed with each of the used vehicles: six rats received by an intra-articular (i.a.) injection 0.05 ml of uric acid suspended in mineral oil in the knee joint of the right hind limb; six rats received by an i.a. injection 0.05 ml of mineral oil (vehicle of uric acid); six rats received vehicle of rofecoxib/tramadol (carboxymethylcellulose 0.5% p.o.), but no uric acid; six rats received by an i.a. injection 0.05 ml of uric acid in the knee joint of the right hind limb, and then 2:50 h, received carboxymethylcellulose 0.5% p.o. At the end of the experiment the rats were immediately euthanized; this action avoided unnecessary suffering to the animals.

2.5. Measurement of gastrointestinal side-effects

Female Wistar rats (150–180 g of body weight) were fasted 24 h before the experiments. Indomethacin (20 mg/kg p.o.) was given to produce 100% gastric ulcers (Lee et al., 1971; Déciga-Campos et al., 2003). Rofecoxib (17.8 mg/kg p.o.), tramadol (10.0 mg/kg p.o.), vehicle (carboxymethylcellulose of 0.5% p.o.) and the combination of rofecoxib plus tramadol (17.8 and 10.0 mg/kg respectively) were administered orally at the same time to five groups (six rats each). About 2.5 h later, all the groups received a second administration of the same doses. Stomachs were examined 5 h after the first treatment as follows: the animals were killed and the stomachs were removed, opened along the smaller curvature, gently rinsed under formol (2%), and examined. The severity of gastric lesions induced by the drug treatments was calculated as the ratio between the number of lesions (stomach ulcer or erosion) caused by a given treatment and the number of lesions produced by indomethacin (100%). This was considered to reflect drug-induced adverse effects. The length in mm of each lesion was measured under a dissecting microscope and the sum of the length of all lesions was designated as the ulcer index. Gastric injury percent was calculated as:

$$\%\textit{Gastric Injury} = \left(\text{mm}^2 \text{ ulcers in treated groups}\right)(100) \div \left(\text{mm}^2 \text{ ulcers in indomethacin group}\right)$$

2.6. Data presentation and statistical evaluation

Data in the text, tables and figures are expressed as the FI%. Curves for FI% vs time were made for each treatment and the corresponding time course was obtained. Antinociception was estimated as the recovery of the FI%. The cumulative antinociceptive effect during the whole observation period (4 h) was determined as the area under the curve (AUC) of the time course to obtain the dose–response curve and to analyze the whole antinociceptive effect elicited by the analgesic agent, either alone or in combination.

The interaction between rofecoxib and tramadol was calculated with surface of synergistic interaction (SSI) analysis (López-Muñoz, 1994) and an isobolographic method (Tallarida et al., 1989). The AUC was calculated for each of the drug combinations and for each of the components. On the basis of the addition of the effects of the individual component drugs (Seegers et al., 1981), an AUC equivalent to the sum was expected. If the sum of the corresponding individual AUCs was higher than the theoretical sum, the result was considered to show potentiation; if it was similar to the theoretical sum, it was considered to show an additive antinociceptive effect; but it was lower to the theoretical sum, it was considered to show sub-additive interaction. The AUC was obtained by the trapezoidal rule (Rowland and Tozer, 1989). All values for each treatment are means ± SEM for six animals. The AUC values for drug combinations were compared with the expected value using Student's *t*-test. The AUC values obtained from the antinociceptive effects produced by either rofecoxib or tramadol (assayed separately) were compared with the AUC value obtained from the corresponding combination by analysis of variance (ANOVA) and Dunnett's test. The gastrointestinal side-effects produced by either rofecoxib or tramadol (assayed either separately or in combination) were compared with the gastrointestinal side-effects obtained from indomethacin by ANOVA and Dunnett's test. $P<0.05$ was considered statistically significant.

The isobologram was constructed using ED_{50} (calculated of the maximal effect reached by each compound) when the drugs were given alone or in combination. To perform the isobolographic analysis, rofecoxib and tramadol were administered orally in combination as fixed ratio proportions of the equieffective ED_{50} dose for rofecoxib and tramadol (1:1). The ED_{50} values (±SEM) for rofecoxib and tramadol alone were plotted on the *x*- and *y*-axes, respectively, and the theoretical additive point was calculated according to Tallarida et al. (1989). From the dose–response curve of the combined drugs, the ED_{50} value of the total dose of the combination was calculated. Statistical significance of the difference between the theoretical additive point and the experimentally derived ED_{50} value was evaluated using Student's *t*-test. An experimental ED_{50} significantly greater

than the theoretical additive ED_{50} ($P<0.05$) was considered to indicate a sub-additive interaction between rofecoxib and tramadol.

3. Results

3.1. Effect of uric acid and vehicles

Uric acid induced complete dysfunction of the right hind limb corresponding to a FI% value of zero in 2.5 h. This dysfunction was maintained throughout the entire experimental period, which lasted another 4 h. Control rats injected with 0.05 ml of mineral oil did not show any significant decrease ($P>0.05$) of FI% during the whole period of observation. The rats that received vehicle of rofecoxib/tramadol (carboxymethylcellulose 0.5% p.o.), but no uric acid, did not show any significant decrease of FI% during the whole period of observation. The rats that received uric acid (i.a.) and then vehicle (carboxymethylcellulose 0.5% p.o.) did not show any significant recovery of the FI% during the observation period. At the doses used, rofecoxib (1.0–56.2 mg/kg) and tramadol (1.8–56.2 mg/kg) did not affect neither the walking ability or produce any motor impairment in the rats during the period of evaluation, as compared with that of the vehicle-treated rats (data not shown). The stainless steel cylinder used in the PIFIR model (which was rotated at 4 rpm, forcing the rats to walk), was employed, as a Rota-rod, to evaluate the possible occurrence of non-specific effects (by example, alterations of the motor coordination, muscle-relaxation or sedation) produced by the analgesic drugs.

3.2. Antinociceptive effects of drugs assayed individually

The oral administration of rofecoxib and tramadol produced dose-dependent antinociceptive effect, as there was significant reduction in the dysfunction as compared to control animals in the algesiometric model. Fig. 1 shows the dose–response curves for each drugs. Both drugs increased AUC in a dose-dependent manner but displayed different efficacy (i.e. rofecoxib produced the maximum effect). Thus, rofecoxib (17.8 mg/kg) showed a great antinociceptive efficacy of 280.2±17.6 area units (au) and tramadol (56.2 mg/kg) showed 179.2±16.0 au. Potency refers to the amount (mg) of drug needed to produce a level of effect. ED_{33} (effective dose estimated to produce 33% or 125 au of the maximum possible effect in the PIFIR model: 375 au) with its SEM using standard linear regression analysis of log dose–response curve was calculated for each drug and used to compare potency analgesic. The ED_{33} values for the drugs indicate that there were significant differences in their antinociceptive potencies: rofecoxib (ED_{33}=5.5±1.2 mg/kg) was more potent than tramadol (ED_{33}=20.3±1.3 mg/kg). There were no adverse effects with the doses used.

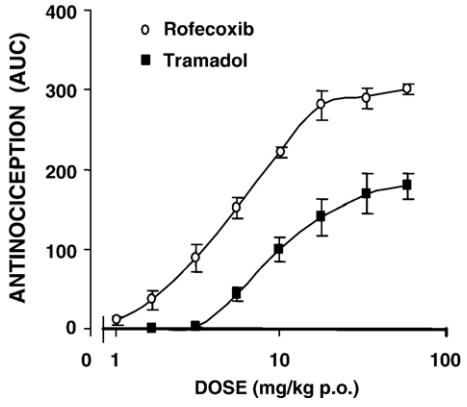

Fig. 1. Oral antinociceptive effect of rofecoxib and tramadol in the pain-induced functional impairment model. The antinociceptive response is expressed on the *y*-axis as the area under the curve (AUC) of the functionality index over the 4-h observation period (% h). Data are expressed as means±SEM for six animals.

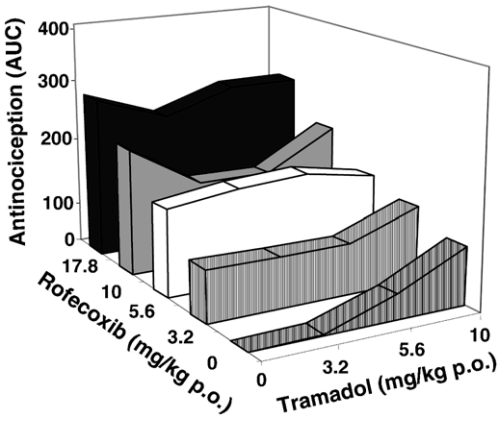

Fig. 2. Antinociceptive effects obtained with rofecoxib and tramadol either alone or in combination. The *y*-axis represents the AUC of the time course; the *x*-axis depicts the doses (mg/kg) of rofecoxib administered simultaneously with tramadol; and the *z*-axis depicts the doses (mg/kg) of tramadol used to obtain the dose–response curves (DRC). The combination rofecoxib (17.8 mg/kg)+tramadol (10.0 mg/kg) showed the greatest antinociceptive effect. Each point represents the mean of 6 experiments; there is an interaction between rofecoxib and tramadol ($P<0.05$).

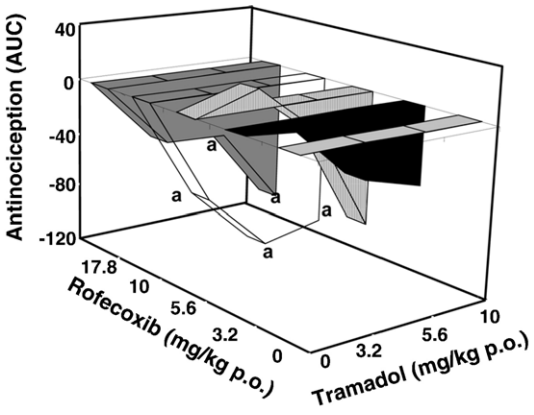

Fig. 3. The antinociceptive effects produced by the different combinations of rofecoxib and tramadol after subtracting the individual effects. The axes are the same as those in Fig. 2. The doses producing either addition or sub-addition when co-administered could be determined. Five results correspond to sub-additive antinociceptive effects ($^aP<0.02$), whereas the other 7 combinations represent addition of antinociceptive effect. Each interaction is represented by the mean for six animals.

Table 1
Comparison of the antinociceptive effects expressed as AUC and FI% produced by some combinations and those produced by the maximal dose of each analgesic drug (rofecoxib or tramadol)

Treatment	Dose (mg/kg)	AUC [a] (au)	Time course curve [b]		
			E_{max} (FI%)	TE_{max} (h) [c]	E_{4h} (FI%) [d]
Antinociceptive effects produced by the maximal dose of each analgesic drug					
Rofecoxib	17.8	280.2±17.6	79.2±5.3	1.50	63.4±10.9
Tramadol	31.2	169.7±25.3	52.8±15.6	1.50	45.5±14.8
Combination that produce the maximum antinociceptive effect					
Rofecoxib	17.8	280.2±17.6	79.2±5.3	1.50	63.4±10.9
Tramadol	10	99.3±15.8	35.1±1.8	1.50	17.7±4.3
Combination		275.4±33.2 [e]	82.2±30.1	0.50	72.3±7.4
Rofecoxib	10	219.7±5.4	60.6±7.2	1.00	59.2±7.4
Tramadol	31.2	169.7±25.3	52.8±15.6	1.50	45.5±14.8
Combination		279.6±31.5 [e]	78.4±5.1	0.50	65.9±10.3
Rofecoxib	17.8	280.2±17.6	79.2±5.3	1.50	63.4±10.9
Tramadol	31.2	169.7±25.3	52.8±15.6	1.50	45.5±14.8
Combination		306.6±17.0 [e]	91.1±2.6	0.50	68.0±6.6
Combinations that produced high sub-additive effect					
Rofecoxib	10	219.7±5.4	60.6±7.2	1.00	59.2±7.4
Tramadol	5.6	43.9±9.4	12.2±3.7	1.00	12.3±5.2
Combination		143.8±12.7 [e]	40.8±7.9	1.00	33.2±14.5
Rofecoxib	10	219.7±5.4	60.6±7.2	1.00	59.2±7.4
Tramadol	10	99.3±15.8	35.1±1.8	1.50	17.7±4.3
Combination		208.7±32.1 [e]	60.5±9.8	1.50	52.5±9.0
Rofecoxib	17.8	280.2±17.6	79.2±5.3	1.50	63.4±10.9
Tramadol	5.6	43.9±9.4	12.2±3.7	1.00	12.3±5.2
Combination		190.9±27.6 [e]	39.3±9.2	1.00	67.9±20.6

[a] Area under the curve of the time course or the whole antinociceptive effect showed for the analgesic drug during the 4-h period, either alone or in combination.
[b] Variables measured for curves of time course.
[c] Time to produce the maximal effect measured for curve of time course.
[d] This FI expresses the antinociceptive effect obtained exactly 4 h after administration; this is the last evaluation of antinociception in the experimental protocol.
[e] $P<0.002$.

3.3. Antinociceptive effects of the drug combinations

Figs. 2 and 3 depict the antinociceptive effect from the 12 combinations on three-dimensional graphs. These were constructed using the mean from six animals for each dose either alone or in combination. The maximal antinociceptive effect attainable from several rofecoxib+tramadol combinations (17.8+10.0 mg/kg, respectively, see Fig. 2) was 275.4±33.2 au. Statistical analysis of data from Fig. 2 indicates an interaction between rofecoxib and tramadol (rofecoxib $F=10.612$, $P<0.01$; tramadol $F=16.733$ $P<0.01$; and rofecoxib+tramadol $F=3.435$ $P<0.05$), whereas there were sub-additive interaction of the combinations tested.

Fig. 3 was produced with the objective of discerning additive from sub-additive effects. This graph was calculated from the total antinociceptive effect produced by the combinations after subtraction of the antinociceptive effect produced by each component alone. Results lower than level "0" were considered to indicate sub-additive interaction, whereas those at level "0" were considered to indicate addition. Although this type of plot allows potentiation antinociceptive effects to be observed, these were not obtained in the present study. Likewise, 7 combinations of rofecoxib+tramadol produced additive antinociceptive effects, and 5 produced sub-additive effects ($P<0.002$; $Q=9.5$) (Fig. 3). These combinations were: 10.0+3.2, 10.0+5.6, 17.8+5.6, 10.0+10.0 and 17.8+10.0 mg/kg of rofecoxib+tramadol respectively. Using this graph it is easy to visualise the drug interactions of rofecoxib+tramadol (i.e. additive or sub-additive effects). For example, 5 combinations of rofecoxib+tramadol displayed various degrees of sub-additive antinociceptive effects, but 3 combinations rofecoxib+tramadol showed high sub-additive interaction (10.0+5.6, 10.0+10.0 and 17.8+5.6 mg/kg respectively), these are shown in Table 1. Tramadol, at a dose of 5.6 mg/kg, yielded an AUC of 43.0±9.4 au and rofecoxib, at the dose of 17.8 mg/kg, rendered an AUC of 280.2±17.6 au; however, the combination of rofecoxib+tramadol (17.8+5.6 mg/kg) yielded an AUC of 190.9±27.6 au, which is lower than the expected AUC resulting from the sum of the individual values i.e. 324.1 au ($P<0.002$; $Q=7.3$). The analysis of the E_{max} from the corresponding time course curves showed a decrease in the values obtained from the combination (39.3±9.2%), which were lower than the corresponding values (rofecoxib 79.2±5.3% and tramadol 12.2±3.7%). The 2 new combinations of rofecoxib (10.0 and 17.8 mg/kg) and tramadol (31.6 mg/kg) showed sub-additive effects too. Other examples of sub-additive effects produced by rofecoxib+tramadol are shown in Table 1.

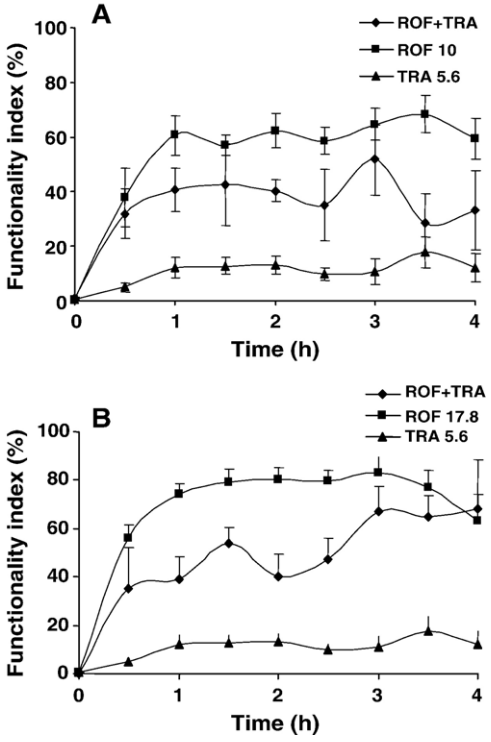

Fig. 4. Time courses of two combinations that produced sub-additive effects: (A) 10.0 mg/kg rofecoxib (■), 5.6 mg/kg tramadol (▲), and the combination of rofecoxib+tramadol (10.0+5.6 mg/kg) (◆); and (B) 17.8 mg/kg rofecoxib (■), 5.6 mg/kg tramadol (▲), and the combination of rofecoxib+tramadol (17.8+5.6 mg/kg) (◆). This latter combination represents a clear example of sub-additive antinociceptive effects; the AUC (190.9±27.6 au) obtained with this combination was lower ($P<0.002$) than the AUC obtained from the sum of the individual AUCs (280.2±17.6 au+43.9±9.4 au). Data are expressed as the means±SEM of 6 determinations.

The antinociceptive effects produced by two combinations that produced sub-additive antinociceptive effects (10.0 mg/kg rofecoxib+5.6 mg/kg tramadol, and 17.8 mg/kg rofecoxib +5.6 mg/kg tramadol) are shown in Fig. 4. As can be seen in Fig. 4A, the antinociception produced by rofecoxib+tramadol (10.0+5.6 mg/kg) represented sub-additive antinociceptive effect obtained with 143.8±12.7 au, while rofecoxib alone (10.0 mg/kg) showed an AUC of 219.7±5.4 au and tramadol alone (5.6 mg/kg) produced 43.9±9.4 au only. The combination depicted in Fig. 4B (17.8 mg/kg rofecoxib+5.6 mg/kg tramadol) represents a combination that produced sub-additive antinociceptive effect (40.9 less AUC or whole antinociceptive effect than the sum of individual AUCs); likewise, both the time course and AUC obtained with this combination were lower ($P<0.002$; $Q=8.6$) than the respective values obtained with the sum of individual agents (324.1 au). The antinociception produced by rofecoxib+tramadol (17.8+5.6 mg/kg) was 190.9±27.6 au; while rofecoxib alone (17.8 mg/kg) showed an AUC of 280.2± 17.6 au and tramadol alone (5.6 mg/kg) produced 43.9±9.4 au only. This result was important if it is considered that the rofecoxib alone (17.8 mg/kg) produced more antinociceptive effect (46.8%) than the combination used.

Another approach for investigating the interaction between the two selected analgesic drugs is the isobolographic method (Tallarida et al., 1989). Isobolographic analysis, using fixed ratio (1:1) ED_{50} revealed a significant sub-additive interaction between rofecoxib and tramadol after oral administration in the pain-induced functional impairment model in the rat, this is shown in Fig. 5. Horizontal and vertical bars indicate SEM. The oblique line between the *x*- and *y*-axes is the theoretical additive line. The point for experimental ED_{50} for this combination was obtained above the theoretical additive line indicating possible sub-additive interaction ($P<0.05$; $Q=2.8$). Further, the experimental ED_{50} dose was significantly higher than the calculated additive ED_{50} doses thereby demonstrating a sub-additive interaction.

ED_{50} (calculated of the maximal effect reached by each compound) with its SEM was calculated for each drug: rofecoxib ($ED_{50}=6.4\pm1.1$ mg/kg) and tramadol $ED_{50}=11.2\pm1.3$ mg/kg.

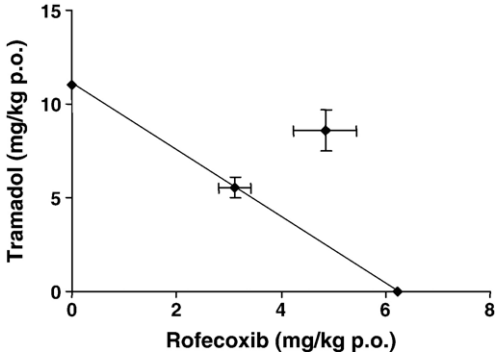

Fig. 5. Isobologram showing the antinociceptive interaction of rofecoxib ($ED_{50}=6.4$ mg/kg) and tramadol ($ED_{50}=11.2$ mg/kg) in the PIFIR model. Horizontal and vertical bars indicate SEM. The oblique line between the *x*- and *y*-axes is the theoretical additive line. The point in the middle of this line is the theoretical additive point calculated from the separate ED_{50} values. The experimental point lies far above the additive line, indicating significant sub-additive effect ($P<0.05$).

Table 2
Effects of indomethacin, rofecoxib, tramadol and rofecoxib+tramadol combination on gastric injure in rats

Treatment	Number of erosions	Ulcers (mm)	Gastric injury (%)
Vehicle	0	0	0
Indomethacin (20 mg/kg)	30.4±4.0	51.6±3.4	100
Rofecoxib (17.8 mg/kg)	18.2±2.1 *	10.7±0.8 *	21.2
Tramadol (10 mg/kg)	0	0.6±0.3	1.9
Rofecoxib+tramadol (17.8+10 mg/kg)	10.8±1.3 *	21.2±2.6 *	40.4

Note: Data expressed as mean±SEM ($n=6$); Gastric injury (%)=ulcer formation expressed as percentage.
* Significantly different from the indomethacin group ($P<0.05$).

3.4. Measurement of gastrointestinal side-effects

The administration of tramadol did not produce ulcers or erosions. Its adverse effects were similar to those of vehicle. However, rofecoxib generated a lower area of ulcers (10.7± 0.8 mm^2) and less number of erosions (18.2±2.1) than did indomethacin ($P<0.001$; $F=23.181$), which was considered to be the most detrimental compound in terms of the number and severity of the lesions caused in the stomach (i.e. ulcers or erosions) (100%). The combination rofecoxib+tramadol generated less ulcers (21.2±2.6 mm^2) and less number of erosions (10.8±1.3) than did indomethacin ($P<0.001$; $F=11.05$), but generated more ulcers (21.2±2.6 mm^2) and less number of erosions (10.8±1.3) than did rofecoxib alone. Interestingly, the combination rofecoxib+tramadol produced sub-additive antinociceptive effects and increased the gastric injuries (Table 2).

4. Discussion

Some reports have been published on analgesic combinations (López-Muñoz et al., 1993a, 1994a,b; López-Muñoz, 1994; Salazar et al., 1995; Maves et al., 1994; Sandrini et al., 1998, 1999; Taylor et al., 1998; Déciga-Campos et al., 2003 ; López-Muñoz et al., 2004). Therefore, the focus of this study was to first examine the antinociceptive efficacy of either rofecoxib or tramadol during arthritic pain, and second, to quantitatively evaluate the antinociceptive interaction between rofecoxib and tramadol. The clinical implications of this study are important, given the desire to maximize analgesia while minimizing adverse effects in a variety of situations in which arthritic pain is a major problem.

This is the first study analyzing the effects of combinations of rofecoxib and tramadol using a model of arthritic pain. The reason for using tramadol was that it is an atypical opioid, whereas rofecoxib is a classical preferential inhibitor of COX-2. The PIFIR model was used because it allows the evaluation of the time course of the antinociceptive effect in the same animal; furthermore, it does not generate conditioned learning and has high sensitivity (López-Muñoz et al., 1993b). The doses used for obtaining the dose–response curve of either rofecoxib or tramadol alone were selected on an increasing 0.25 logarithmic units basis. The doses used for analyzing the combinations (surface of synergistic interaction analysis) were selected from

the respective dose–response curves. The 12 different associations were planned using 4 doses of the preferential inhibitor of COX-2 with 3 low doses of the morphine-like drug: tramadol. This allowed for the detection of the profile of antinociceptive interaction between the various combinations. The doses used to analyze the effects of the combinations were selected on the basis of their lack of adverse effects when administered alone. Because, using combinations of medications that offer analgesic synergism could allow a reduction in required dosage and decrease the incidence of adverse effects, in our experiments were used for rofecoxib dose range that covers the whole field, but the dose selected for tramadol (the opioid drug) was in the low intrinsic range (doses up to 10 mg/kg were used). On account of this, were included 2 new combinations, these were of rofecoxib 10 and 17.8 mg/kg with tramadol 31.6 mg/kg (doses down to 10 mg/kg), and the analysis of the results obtained were of sub-additive effects (Table 1).

The surface of synergistic interaction analysis was applied to assess the antinociceptive effects produced by rofecoxib and tramadol, either separately or in different combination ratios, which may allow the determination of the optimal ratio that produces the antinociceptive activity; the isobolographic method, on the other hand, was used to confirm the antagonistic effect. The two methods used to assess synergistic interaction between antinociceptive compounds are excellent tools in pharmacology, but it should be highlighted that the purpose of the present study was not related with the comparison of the two methods to assess synergistic interaction between antinociceptive compounds.

Our group has used the surface of synergistic interaction analysis to determine additive (i.e. the sum of the effects produced by the each agent alone), sub-additive (i.e. less than the sum of the effects produced by the each drug alone), or supra-additive (i.e. synergistic; greater than the sum of the effects produced by the each drug alone) interaction, such as morphine + dipyrone (López-Muñoz, 1994), morphine + aspirin (López-Muñoz et al., 1995), D-propoxyphene + aspirin (López-Muñoz, 1995), D-propoxyphene + acetaminophen (López-Muñoz and Salazar, 1995), tramadol + aspirin (Salazar et al., 1995), morphine + rofecoxib (Déciga-Campos et al., 2003), and ketorolac + tramadol (López-Muñoz et al., 2004) all of which were found to produce different degrees of antinociceptive potentiation. This analysis permitted the evaluation and determination of analgesic drug doses that will exert maximal potentiating effects. It is therefore expected that this approach will have significant implications for the treatment of pain (López-Muñoz et al., 2004).

The purpose of analgesic drug combinations is to optimize dose regimens so that greater analgesic effects are obtained with decreased unwanted side-effects. Then, administration of combinations of drugs like morphine with NSAIDs can lead to the use of lower doses of opioid drugs with increased therapeutic effects (Wei-wu et al., 1999; Picard et al., 1997). It has been demonstrated that the combination of morphine with some NSAIDs increases antinociceptive effects and decreases adverse events (Déciga-Campos et al., 2003; López-Muñoz et al., 2004).

The results obtained in this study showed either additive or sub-additive effects between rofecoxib and tramadol; over the dose ranges used, the antinociceptive activities of rofecoxib and tramadol given individually tended to be more efficacious than those observed when they were administered in combination. Similar results were reported using tramadol/rofecoxib in mice (Satyanarayana et al., 2004). Satyanarayana et al., evaluated the combination of naproxen + tramadol, and rofecoxib + tramadol in acetic acid-induced writhing in mice, and the isobolographic analysis indicated synergistic or supra-additive interactions for the combinations of naproxen and tramadol, however similar interaction was not reported when tramadol was combined with rofecoxib. Our results are consistent with the previous report, although, the study design and experimental model were different: in the present study, we examined 12 different combinations, and this study design showed that the co-administration of rofecoxib + tramadol may result in additive (i.e. the sum of the effects produced by the each agent alone), or sub-additive (i.e. less than the sum of the effects produced by the each drug alone) interaction.

There exists a considerable controversy in the literature with regard to the effect of COX-1 and COX-2 inhibition in gastric injury. The use of traditional NSAIDs for the relief of pain and inflammation increases the risk of gastrointestinal side-effects ranging from dyspepsia to symptomatic and complicated ulcers (Hawkey et al., 2003; Hollenz et al., 2006). The use of COX-2-selective agents appears to be little clinically significant difference between COX-2 and traditional NSAIDs in terms of dyspepsia, a common cause of the discontinuation of a traditional NSAIDs (Layton et al., 2003; Watson et al., 2004; Motilva et al., 2005). Otherwise, COX-1 but also COX-2 has important functions in the maintenance of gastric integrity. The maintenance of gastric mucosal function and integrity highly depends on the status of microcirculation (Schmassmann et al., 2006). Vasoactive agents (prostaglandins, nitric oxide, calcitonin gene-related peptide and GABA) play a crucial role in mucosal defensive processes (Ehrlich et al., 2004). On the other hand, tramadol is known to have minimal effect on intestinal transit in healthy volunteers and pain patients (Wilder-Smith and Bettiga, 1997; Wilder-Smith et al., 1999a,b). However, there exist evidences that relate the effect of opiate receptor blockade with the gastroprotection; naloxone dose-dependently protects against the indomethacin- and HCl-, but not against the ethanol-induced gastric mucosal damage; morphine aggravates the HCl-induced ulcerogenesis as well as both opioid receptor agonist and antagonist decrease gastric acid secretion (Debreceni et al., 1997). Therefore, morphine administration substantially attenuated the protective actions of the prostaglandin analogue 16,16 dimethyl prostaglandin E2 against ethanol-induced damage (Esplugues et al., 1992). We performed experiments to evaluate the effects of tramadol (10 mg/kg) and rofecoxib (17.8 mg/kg) co-administration on gastric damage-indomethacin in rat. Our results showed that tramadol and rofecoxib co-administration could increase the incidence of gastrointestinal side-effects (ulcers and erosions) that produced by each drug alone. In addition, the results of sub-additive antinociceptive interaction reflect that the antinociceptive interaction of rofecoxib and tramadol may have not clinical utility in pain therapy. The mechanism of sub-additive effects between rofecoxib and tramadol is difficult to

explain. Further experiments will be required to determine the mechanisms involved in these effects. Clearly, our study was designed to characterize the type of antinociceptive synergism, but not the mechanism of action involved.

One mechanism of action of NSAIDs involves suppression of the synthesis of prostaglandins (Lorenzetti and Ferreira, 1985), and the mediation of the arginine–nitric oxide–cGMP pathway (Duarte et al., 1992). More recent evidence suggests that NSAIDs may also have direct central effects (Burian and Geisslinger, 2005; Dembo et al., 2005; Kwon et al., 2005; Lizarraga and Chambers, 2006). Rofecoxib may partly generate antinociceptive effects through the NO–cyclic GMP pathway (Déciga-Campos and López-Muñoz, 2003) and the serotoninergic system (Déciga-Campos et al., 2004). COX-2 NSAIDs were developed to limit NSAIDs adverse effects, however, while COX-2 therapy may reduce the risk of gastrointestinal ulceration, recent evidence indicates that COX-2 therapy may not reduce the risk of cardiovascular complications (Heim and Broich, 2006; Motsko et al., 2006). Tramadol, the atypical opioid analgesic, is a racemic mixture of two enantiomers with (−)-tramadol preferentially inhibits noradrenaline (NA) uptake, whereas (+)-tramadol inhibits serotonin (5-HT) uptake, enhances 5-HT release, and binds to mu opiate receptors (Berrocoso et al., 2006). It is rapidly and extensively absorbed after oral doses and is metabolized in the liver (Lewis and Han, 1997). Probably the mucosal damage induced by the COX-2 inhibitor (rofecoxib), which inhibit prostaglandin gastroprotection, increase the damage when tramadol occupants the opioid receptors. There are evidences which show that the actions of morphine in reducing prostaglandin protection against mucosal injury were abolished by pretreatment with naloxone or the peripherally acting opioid antagonist, *N*-methyl nalorphine (Esplugues et al., 1992).

Previous studies have shown that the combination of morphine with some NSAIDs can activate the serotonergic (Sandrini et al., 1998) and the opioid (Maves et al., 1994) systems, and evidence has also been provided for the participation of the nitric oxide–cGMP pathway and other mechanisms such as activation of opioid and prostanoid receptors. It has been proposed that opioids produce analgesia within the midbrain periaqueductal grey by inhibiting gamma-aminobutyric acid (GABA) system on neurones, which form part of a descending antinociceptive pathway, and microinjections of cyclooxygenase inhibitors into the periaqueductal grey produce analgesia (Tortorici and Vanegas, 1995). Vaughan et al. (1997) have hypothesized a mechanism that involves opioid modulation of arachidonic acid metabolites in GABA interneurons. These authors demonstrated that opioids might be coupled to a voltage-dependent potassium conductance via a pathway involving phospholipase A_2, arachidonic acid and 12-lipoxygenase. Cyclooxygenase inhibitors potentiate opioid inhibition of GABA synaptic transmission, presumably because more arachidonic acid is available for enzymatic conversion to 12-lipoxygenase products (Vaughan et al., 1997). Therefore, it was demonstrated that inhibition of cyclooxygenase-1, rather than of cyclooxygenase-2, potentiates the inhibitory action of opioids on GABA synaptic transmission (Vaughan 1998). Previous studies using rofecoxib + morphine (Déciga-Campos et al., 2003) had showed that combinations containing opioid drugs (morphine) and preferential COX-2 inhibitors (rofecoxib) may have clinical utility in pain therapy. In the present study, it was shown that rofecoxib, which is a preferential inhibitor of cyclooxygenase-2, may produce sub-additive interaction with tramadol. (−)-Tramadol preferentially inhibits NA uptake, whereas (+)-tramadol inhibits 5-HT uptake, enhanced 5-HT release, and binds to mu-opioid receptors. In addition, a marked antinociceptive synergy exists between these the two enantiomers (Raffa et al., 1993, Satyanarayana et al., 2004). In the present study, the roles of serotoninergic and adrenergic modulation were not studied. However, other pharmacodynamic/pharmacokinetic interactions cannot be excluded. In addition to possible pharmacodynamic mechanism of the combination antinociception, rofecoxib may alter the pharmacokinetic properties of the tramadol. The authors are not aware of any data that answer this question: however, future studies will address this issue.

In conclusion, the surface of synergistic interaction and isobolographic analysis indicated an additive and sub-additive interaction between the preferential inhibitor of cyclooxygenase-2 (rofecoxib) and tramadol when administered orally during arthritic nociception in the rat. These data showed that: (1) oral rofecoxib is not a potentiator of tramadol antinociception during arthritic nociception in the rat; 2) oral co-administration of rofecoxib and tramadol produced an antinociceptive effect similar or lower than that observed after individual treatment; 3) the sub-additive antinociceptive effects were accompanied by increased side-effects; 4) the fact that the combinations of rofecoxib + tramadol produced many sub-additive effects is interesting; and 5) the antinociceptive interaction of rofecoxib and tramadol suggest that combinations with these class of drugs may have not clinical utility in pain therapy.

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